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The cytoplasmic tail of L-selectin interacts with the adaptor-protein complex AP-1 subunit μ1A via a novel basic binding motif

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ABSTRACT

L-selectin regulates leukocyte adhesion and rolling along the endothelium. Proteins binding to the cytoplasmic tail of L-selectin regulate L-selectin functions. We used L-selectin cytoplasmic tail peptide pulldown assays combined with high sensitivity liquid chromatography/mass spectrometry to identify novel L-selectin tail binding proteins. Incubation of the L-selectin tail with cell extracts from phorbol myristate acetate-stimulated Raw 264.7 macrophages resulted in the binding of μ1A of the clathrin-coated-vesicle AP-1 complex. Furthermore, full length GST-μ1A and the GST-μ1A C-terminal domain but not the GST-μ1A N-terminal domain, bind to L-selectin tail peptide and the intracellular pool of L-selectin colocalizes with AP-1 at the trans-Golgi network. We identified a novel basic protein motif consisting of a cluster of three di-basic residues (RR357, KK360, and KK363) in the membrane-proximal domain of L-selectin tail as well as a doublet of aspartic acid residues (DD370) in the membrane-distal end of L-selectin tail involved in μ1A binding. Stimulation of Raw 264.7 macrophages with PMA augmented the amount of μ1A associated with anti-L-selectin immunoprecipitates. However, full length GST-μ1A did not bind to phospho-L-selectin tail or phospho-mimetic Ser364Asp L-selectin tail. Accordingly, we propose that phosphorylation of μ1A is required for interaction with L-selectin tail and that L-selectin tail phosphorylation may regulate this interaction *in vivo*. Molecular docking of L-selectin tail to μ1A was used to identify the μ1A surface domain binding L-selectin tail and to explain how phosphorylation of L-selectin tail abrogates μ1A interaction. Our findings indicate that L-selectin is transported constitutively by the AP-1 complex leading to the formation of a trans-Golgi network reserve pool and that phosphorylation of L-selectin tail blocks AP-1-dependent retrograde transport of L-selectin.

INTRODUCTION

Leukocyte trafficking is an essential mechanism of immune surveillance. The migration to and from peripheral tissues enables leukocytes to patrol the entire body, providing immune responses as needed. The exit of leukocytes from the circulation and migration into tissues is regulated at the level of cell interactions with the vascular endothelium. At least two families of adhesion receptors participate in this interaction: the integrins and the selectins (1). Selectins mediate the initial phase of leukocyte recognition of endothelium and this takes the form of leukocyte rolling along the vessel wall (1). There are only 3 types of selectins: L, E, and P. L-selectin is expressed exclusively in leukocytes and acts as a major contributor of leukocyte tethering and rolling (2). Indeed, mice deficient in L-selectin display defects in lymphocytes homing to lymphoid organs and in neutrophil accumulation in inflamed tissues (3). Unlike E- and P-selectins, L-selectin is constitutively expressed on the membrane and possesses a membrane-proximal cleavage site that is hydrolysed after leukocyte activation, leading to shedding of its extracellular domain (4). L-selectin shedding plays a key role in the regulation of leukocyte functions. It impairs the homing of activated lymphocytes to lymph nodes (5), augments neutrophil's rolling velocity (4), controls transendothelial migration of monocytes (6) and confers lytic activity to T lymphocytes (7).

L- selectin is composed of an N-terminal lectin domain, binding endothelial ligands, followed by an epidermal growth factor like domain, a short consensus repeat domain, a transmembrane domain, and a short cytoplasmic tail of 17 amino acids (8). The cytoplasmic tail of L-selectin is essential for L-selectin function and localization on the tips of microvilli. Indeed, L-selectin mutants lacking the membrane distal 11 amino acid residues cannot support leukocyte rolling along endothelial venules and adhesion (9). This defect in L-selectin function cannot be explained by changes in ligand binding, but is rather due to a defect in the recruitment of binding partners to L-selectin cytoplasmic tail.

To date, only a limited number of L-selectin tail binding partners are known. These include calmodulin (10), Ezrin-Radixin-Moesin (ERM) family of proteins (11), α-actinin (12), Lck (13), and protein kinase C [1](PKC) family members (14). Regions of the L-selectin tail involved in the interaction with these proteins have been mapped. For instance, calmodulin binds the unusual highly positively charged membrane proximal N-terminal motif of di-basic residues ^{356}RRLKKGKKS^{364}. Moesin binds arginine 357 and lysine 362 of this domain (15). On the other hand, the more acidic C-terminal sequence of 11 amino acids of L-selectin cytoplasmic tail binds the cytoskeletal protein α-actinin (12) and the tyrosine kinase Lck (13), whereas the binding sites for PKC family members have not been identified yet. Binding of moesin to the RRLKK motif facilitates the anchor of L-selectin to microvilli, which is critical for leukocyte adhesion and rolling (15). Calmodulin binding is thought to induce a conformational change in the extracellular lectin domain of L-selectin that renders the cleavage site resistant to proteolysis (10). Interaction of α-actinin with the C-terminal region of the tail is important for leukocyte tethering and rolling, but is not essential for ligand recognition (12). Finally, the recruitment of Lck to the C-terminal tyrosine residue (13) and binding of PKC family members to L-selectin tail (14) are essential for L-selectin outside-in signalling.

Despite this gained knowledge, several aspects of L-selectin functions remain poorly understood. For example, the mechanism controlling the replenishment of L-selectin on the membrane surface and the clustering of L-selectin on the tips of microvilli are not well known. Identification of novel L-selectin cytoplasmic tail interacting proteins may shed light on the mechanisms regulating L-selectin functions.

The murine L-selectin tail contains only one serine residue at position 364, which is conserved in human L-selectin. L-selectin is phosphorylated in response to T lymphocyte receptor cross-linking or treatment with PMA (14) (an activator of PKC) or engagement of chemokine receptors (16). It was proposed, but not proven, that such phosphorylation may be essential for the dynamic association/dissociation of proteins to L-selectin tail relevant to the regulation of L-selectin functions. Indeed, moesin (11) and PKC family members (14) bind to L-selectin tail in lymphocytes stimulated with PMA,

but not in resting cells, whereas calmodulin binds L-selectin tail only in resting cells (10).

In this study, we aimed at identifying novel proteins interacting with L-selectin cytoplasmic tail domain by using sophisticated mass-spectrometry-based proteomics. We report the binding of the μ1A adaptin subunit of the AP-1 complex (17-19) to L-selectin tail and the regulation of this binding by phosphorylation of L-selectin cytoplasmic tail and μ1A. We propose that the AP-1-dependent transport of L-selectin may not be constitutive but regulated by the dynamic interaction between L-selectin tail and μ1A in specific cellular compartments.

RESULTS

Identification of novel proteins interacting with the L-selectin cytoplasmic domain

To identify novel proteins binding to L-selectin cytoplasmic tail, we synthesized a desthiobiotinylated peptide comprising the entire 17 amino acid long cytoplasmic tail of murine L-selectin. As a control, a desthiobiotinylated scrambled peptide of similar size was designed. A linker between the desthiobiotin group and the first amino acid of the two peptides was added (Fig. 1A). This was to ensure that the desthiobiotin group, when coupled to Dynabeads MyOne stretptavidin C1 beads, does not interfere with the binding of proteins to the N-terminal membrane proximal residues of L-selectin tail peptides.

To perform peptide pulldown assays, Raw 264.7 macrophages were stimulated with PMA (1 min, 1μg/ml) and lysed in MPER buffer. Cleared lysates were incubated with either the L-selectin tail peptide (L-sel peptide) or a scrambled peptide (sc peptide) pre-coupled to Dynabeads MyOne streptavidin C1 beads. Cell lysis and experimental conditions (incubation time, washing conditions) were optimized to allow identification of novel L-selectin tail interacting proteins. Our liquid chromatography (LC)/mass spectrometry (MS) analysis was validated by the identification of L-selectin peptides (Sell), which derived from the bait used. We identified four novel proteins interacting specifically with L-selectin tail peptide. These include μ1A-adaptin (AP1m1 of the adaptor-protein complex 1 AP-1, ID: 11767), Cfh (complement component factor h, ID: 12628), Grn (granulin, ID: 14824), and Lrrc48 (leucine rich repeat containing 48, ID: 74665) (Fig. 1B). The highest MS signal intensity associated with L-selectin tail peptide was obtained for μ1A, therefore, we decided to analyse this interaction in more detail.

μ1A is one of the four adaptin subunits of the adaptor-protein complex 1 (AP-1). AP-1 controls the sorting of transmembrane proteins into clathrin-coated transport vesicles, mediating transport between the trans-Golgi network (TGN) and endosomes (17, 18, 20). This way, this vesicular carrier regulates the transport of cargo proteins (including receptors) to the plasma membrane (21).

The C-terminal domain of μ1A interacts with L-selectin

To confirm the μ1A/L-selectin tail interaction, we investigated the binding of full-length μ1A as well as of μ1A subdomains, expressed in *E. coli* as GST-fusion proteins, to L-selectin tail peptide. μ1A consists of a N-terminal domain (aa 1-153), positioned close to the membrane, and a C-terminal domain (aa 154-423), which binds the canonical YxxØ sorting motif (17, 18, 22, 23). Expression of cDNAs encoding for full-length μ1A (GST-FL-μ1A), the C- (GST-C-μ1A) or the N-terminal domain (GST-N-μ1A) of μ1A *in E. coli* resulted in the synthesis of GST-fusion proteins with predicted molecular weights as shown on the polyacrylamide gel stained with Coomassie blue (Fig. 2A).

We next incubated the L-sel peptide, or the sc peptide with purified GST-FL-μ1A. Thereafter, the beads were collected, washed, and re-suspended in Laemelli buffer. Proteins were resolved on 10% SDS-PAGE, transferred on to a PVDF membrane and subjected to Western blot analysis using an anti-GST Ab (Fig. 2B & C) or an anti-μ1A Ab (Fig. 2D). As shown in Fig. 2B, GST-FL-μ1A binds to the L-sel peptide, but not to the sc peptide. GST (added in excess) did not bind to the L-sel peptide, confirming binding specificity. In addition, we found that GST-C-μ1A, but not GST-N-μ1A, binds the L-sel peptide (Fig. 2C, D). Thus the C-terminal domain of μ1A binds L-selectin cytoplasmic tail, as it has been demonstrated for proteins with canonical YxxØ sorting motifs whereas, as expected, the N-terminal domain, which binds the β1 subunit of the AP-1 complex (23), did not bind to L-selectin tail.

μ1A is associated with anti-L-selectin receptor immunoprecipitates

We next sought to characterize the interaction between μ1A, as part of the AP-1 complex, and L-selectin tail in cells in culture. To this end, Raw 264.7 macrophages were stimulated or not for different time periods with PMA (1 μg/ml), before

being lysed. Cell lysates were incubated with either an anti-L-selectin Ab or a control isotype-matched IgG2a. μ1A (AP-1) association with anti-L-selectin immunoprecipitates was investigated by Western blot analysis using an anti-μ1A Ab.
We found little association between L-selectin and μ1A (AP-1) at steady sate in resting cells. However, we observed a time-dependent increase in the amount of μ1A (AP-1) associated with anti-L-selectin immunoprecipitates upon stimulation of the cells with PMA (1 μg/ml) (Fig. 3A, top panel). As a control, we showed that μ1A (AP-1) is not associated with IgG2a immunoprecipitates (Fig. 3B, top panel). We also verified that anti-L-selectin Abs precipitated L-selectin (Figs. 3A and B, bottom panels) as evidenced by the detection of proteins with molecular weights of 75-120 kDa representing the different glycosylated forms of L-selectin (24). However, due to its low level of expression in Raw 264.7 macrophages, the presence of L-selectin in crude cell lysates could not be detected under these experimental conditions.

L-selectin and AP-1 in-vivo colocalization

To test for *in-vivo* L-selectin and AP-1 colocalization by confocal microscopy, we used an established THP-1 cell line expressing wild-type, full-length L-selectin green-fluorescent-protein (GFP) (6). Endogenous AP-1 was labeled with an antibody directed against the γ1 adaptin AP-1 subunit (20). This antibody recognizes the C-terminal flexible region of γ1, which is even accessible when AP-1 is part of a membrane coat. Actin staining (red) served as control. Images show two pools of L-selectin (green), one at the plasma membrane and a large intracellular pool (Fig. 4 a & b). The intracellular pool is clustered around large membranes domains which have AP-1 bound (blue) and therefore represent TGN membranes.
Mander's colocalization coefficient was measured to prove L-selectin/μ1A (AP-1) colocalization in intracellular vesicles (overlap coefficient between blue and green signals). We compared the colocalization coefficient measured in an area including intracellular vesicles to the colocalization coefficient measured in an area containing the plasma membrane (Fig. 4b). Since there is no AP-1 at the plasma membrane, the colocalization coefficient measured in this area is not a real colocalisation signal and is used as a background reference.
We found that the colocalization coefficients were 0.80 ± 0.09 and 0.15 ± 0.01 for intracellular vesicles and plasma membrane areas, respectively (Fig. 4c) ($P<0.0001$). This result demonstrates that L-selectin and AP-1 (μ1A) colocalize in intracellular vesicles.
The limited L-selectin/AP-1 colocalization is in line with the transient AP-1/cargo protein interactions during protein sorting and transport via CCV. Thus, TGN-associated L-selectin may serve as a reserve pool. These proteins will be transported to the plasma membrane when this pool needs to be replenished. One function of AP-1 is the sorting of mannose 6-phosphate receptors (MPR), which facilitate the transport of soluble lysosomal enzymes from the TGN to endosomes. AP-1-dependent MPR sorting ensures that 50% of the cellular MPR pool resides in the TGN (awaiting newly synthesized lysosomal enzymes) whereas only 10% of the cellular pool is at the plasma membrane due to their highly efficient AP-2 mediated clathrin-dependent endocytosis (20, 21). Thus, AP-1-dependent L-selectin sorting might be responsible for the formation of a localized L-selectin reserve pool in the TGN.

Phosphorylation of Ser364 of the L-selectin tail peptide prevents binding of μ1A

To investigate whether phosphorylation of serine 364 of L-selectin tail affects μ1A binding, we synthesized a L-selectin tail peptide, in which serine 364 was replaced by a phospho-serine (L-sel-p-peptide). We found that GST-FL-μ1A, GST-C-μ1A or murine μ1A (AP-1) from cell lysates bind the L-sel peptide, but not the L-sel-p-peptide (Fig. 5A). We also designed a L-selectin peptide in which serine 364 was replaced by an aspartic acid residue (L-sel S364D) to imitate a constitutively phosphorylated L-selectin tail. Again, we found that GST-FL-μ1A, GST-C-μ1A or murine μ1A (AP-1) from a cell lysate bind the L-sel peptide, but not the L-sel S364D peptide (Fig. 5A). To ensure that this effect was due to the introduction of a negative charge at position 364 of L-selectin tail and not to the replacement of serine 364 by

another amino acid, we also conducted experiments with a control peptide in which serine 364 was replaced by an alanine residue (L-sel S364A). We found that GST-FL-μ1A (Fig. 5B, left panel) or GST-C-μ1A (Fig. 5B, right panel) bound equally well to L-sel and L-sel S364A peptides. Thus, we concluded that phosphorylation of L-selectin tail on serine 364 abrogates its interaction with μ1A (AP-1).

Clusters of positively charged amino acids are essential for μ1A interaction with L-selectin tail

The membrane proximal domain of L-selectin tail contains an unusual cluster of 3 di-basic (positively charged) amino acids, R356R357, K359K360 and K362K363. To investigate if this RRLKKGKK sequence of L-selectin tail is involved in the interaction with μ1A, we designed peptides in which one arginine (R) or one lysine (K) residue within any of the 3 di-basic motifs were replaced by an alanine (A) residue (Fig. 6A). These peptides were incubated with GST-FL-μ1A or GST-C-μ1A and an interaction between the proteins and peptides was analysed as described above.

We found that replacing a single arginine (R) or a single lysine (K) residue within any of the 3 di-basic clusters was sufficient to prevent GST-FL-μ1A (Fig. 6B, left) or GST-C-μ1A (Fig. 6B, right) to interact with L-selectin tail. We also investigated whether the C-terminal, negatively charged, aspartic acid (D) residues D369D370 of L-selectin tail have a function in μ1A binding. We replaced separately each aspartic acid residue by an asparagine residue (D369N or D370N) or both were substituted with asparagine residues (D369ND370N) (Fig. 6C). We found that replacement of one or two aspartic acid residues by asparagine augmented the binding of GST-C-μ1A to these L-selectin mutated tails (Fig. 6D). Thus, the highly positively charged membrane proximal region of the L-selectin tail is essential for μ1A binding, whereas its membrane distal part, with its doublet of negatively charged amino acids, weakens the interaction with μ1A.

Molecular docking of the L-selectin tail to μ1A

To identify the μ1A surface domain binding L-selectin cytoplasmic tail and to interpret the results of μ1A binding to L-selectin tail with substituted amino acids, we explored docking of L-selectin tail to the crystal structure of μ1A, as determined in the tetrameric AP-1 complex. To do this, we took into consideration the fact that the L-selectin tail binding surface domain of μ1A has to be accessible and not sterically blocked by the interactions of μ1A with the other subunits of the AP-1 complex. Figure 7A shows the favourable docking pose, which explains the binding specificities of μ1A to L-selectin tail peptides. The L-selectin tail-μ1A interaction is predicted to have a strong electrostatic nature arisen by two complementary charged surface areas at the binding interface. The positively charged cluster of arginine and lysine residues of the N-terminal region of L-selectin tail are matched by several negatively charged residues, namely the aspartate and glutamate residues E311, E337, E340 and D417 of μ1A. In addition, the positively charged residues of L-selectin tail form hydrogen bonds with residues N318, N338 and Q419 of μ1A. The interactions cited above are disrupted when the positively charged residues of the RRLKKGKK motif of L-selectin tail are substituted to alanine and this explains the loss of μ1A binding. S364 of L-selectin tail does not form a specific interaction with μ1A, in line with the unaltered binding of the S364A tail to μ1A, but it is in close proximity to the negatively charged residues E311 and E340 of μ1A. Therefore, introducing a negatively charged group at position 364 of L-selectin tail by addition of a phosphate group or by substituting serine 364 by an aspartic acid residue, will cause electrostatic repulsion, thus preventing binding of μ1A to L-selectin tail.

The aspartic acid residues at positions 369 and 370 of L-selectin tail form polar interactions with K199 and R410 of μ1A, respectively, which would stabilize the binding of μ1A. However, K199 and R410 have also polar interactions with E261 and D174 of μ1A. The aspartate residues at positions 369 and 370 of L-selectin tail could have some repulsion with E261 and D174 as well as the nearby E381, which would weaken the binding. Asparagine residues at these positions could form interactions not only with the positively charged K199 or R410, but also with the negatively

charged E261, D174 or E381 residues of μ1A, thereby enhancing the binding of the two proteins. This explains the augmented binding of μ1A to L-selectin tail peptides in which the aspartic acid residues 369 and 370 were replaced by asparagine residues. Our predicted binding mode is in good agreement with our biochemical binding experiments.

Since μ1A (AP-1) and μ2 (AP-2) are highly homologous and because both bind YxxØ-based sorting motifs and have overlapping cargo specificities, we also modelled the L-selectin tail onto the C-terminal μ2 domain to look if a binding mode could permit an interaction, even though μ2 was not identified in our L-selectin tail pulldown experiments (Fig. 1). Docking predicts the interaction energy of 150-275 kcal/mol for L-selectin/μ2 binding poses, contrasting 400-500 kcal/mol for L-selectin/μ1A binding poses. Thus interactions of L-selectin tail with μ2 are much less favourable confirming the pulldown experiment. It is likely that L-selectin is not endocytosed by the AP-1 homologous plasma membrane AP-2 complex.

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DISCUSSION

In this study, we performed L-selectin cytoplasmic tail pulldown assays combined with high sensitivity LC/MS platform to identify novel L-selectin tail binding proteins in cell extracts from stimulated Raw 264.7 macrophages. By using LC/MS-based peptide sequencing, we identified the AP-1 adaptor-protein complex subunit μ1A as a novel binding protein of L-selectin tail. We confirmed this interaction by showing that: 1) μ1A expressed as a GST fusion protein binds to L-selectin tail; 2) μ1A as part of a fully assembled AP-1 complex is associated with anti L-selectin immunoprecipitates; 3) L-selectin is present as an intracellular pool, which colocalizes with AP-1; 4) The C-terminal μ1A domain binds selectively the non-phosphorylated L-selectin tail.

μ1A is one of four adaptin subunits of the AP-1 complex. AP-1 selects the cargo proteins for clathrin-coated vesicles (CCV) and it coordinates CCV formation as well as CCV uncoating, a prerequisite for vesicle-organelle membrane fusion. Besides μ1A, the AP-1 complex contains the adaptin subunits γ, β1 and σ1A (18, 19). To incorporate cargo proteins into transport vesicles, μ1A binds to specific sequences (called sorting motifs or cargo-binding domains) within the cytoplasmic domains of such proteins. μ1A is composed of two domains. The N-terminal domain (aa 1-153) binds the β1 subunit of the AP-1 complex and thus contributes to the assembly of the AP-1 complex. The C-terminal μ1A domain (aa 153-422) binds sorting motifs of cargo proteins incorporated into CCV (23). In keeping with these structure/functions studies of the AP-1 complex, we discovered that GST-C-μ1A but not GST-N-μ1A binds to L-selectin tail.

Two canonical sorting motifs in cytosolic tails of transmembrane proteins have been described for AP-1: the tyrosine-based motif YxxØ (Ø is a bulky hydrophobic residue), which is bound by the μ1A C-terminal domain and the dileucine based motifs (e.g. D/E]XXXL[L/I), which are bound by σ1A and/or σ1B adaptins (25, 26). L-selectin cytoplasmic tail does not contain either of these two sorting motifs. This means that other sequences must be involved in the interaction with μ1A.

In addition to these two canonical motifs, non-canonical binding motifs have been identified which interact with AP-1 as well. For example, yeast AP-1 binds a 12-residue domain of the pheromone receptor Ste13p, which contains a tribasic sequence KRK (27). In addition, AP-1 binds to the highly basic RARHRRNVDR sequence of the polymeric immunoglobulin receptor (28). Based on these examples, the 3 di-basic clusters of amino acids in L-selectin tail most likely represent a novel, basic, non-canonical binding motif sequence for μ1A. To investigate this possibility, we designed L-selectin tail peptides in which one arginine or lysine residue within any of the 3 di-basic motifs were replaced by alanine residues (R357A, K360A, K363A). We showed that GST-FL-μ1A or GST-C-μ1A did not bind R357A, K360A, or K363A L-selectin tail peptides. This experiment demonstrated that each di-basic residue within the N-terminal membrane proximal region of L-selectin tail is essential for binding μ1A. To understand the role of residues R357, K360, and K363 for μ1A interaction, we used molecular docking to predict the predominant binding mode of L-selectin tail with the 3D structure of μ1A. We found that the positively charged cluster of arginine and lysine residues of the N-terminal region of L-selectin tail ^{356}RRLKKGKKS^{364} are matched by aspartate and glutamate residues E311, E337, E340 and D417 of μ1A and form hydrogen bonds with residues N318, N338 and Q419 of μ1A. While this L-selectin tail region also binds moesin/ezrin, it has been shown that only R357, but not K360 or K363, is required for this interaction (15). Thus, the motifs mediating the binding of moesin/ezrin and μ1A overlap, but involve different amino acid residues. It was reported that association of calmodulin with L-selectin tail is influenced by the membrane bilayer. Thus, in a phospholipid bilayer (which mimics the inner leaflet of the plasma membrane), the positively charged N-terminal part of L-selectin tail interacts with anionic phosphatidylserine lipids at the membrane interface through electrostatic interactions. This prevents calmodulin to interact with the N-terminal part of L-selectin tail (29) and promotes L-selectin shedding (15). Disruption of the interaction between the positively charged N-terminal part of L-selectin tail with the

phospholipid bilayer is a prerequisite for the binding of calmodulin to L-selectin tail. Such disruption is brought about by the interaction of the FERM domain of moesin with L-selectin to induce a separation of the cationic N-terminal part of L-selectin tail from the phospholipids (30). We don't know if the phospholipid bilayer plays a similar role in intact cells for at least two reasons. First, the lipid bilayer, is itself a complex mixture of lipids. Its structure in the plane of the bilayer is that of a microscopic mosaic of regions that differ in composition, that is, domains or clusters (31). In particular, receptors are found in domains such as caveola which are rich in cholesterol. Second, the cited authors (29, 30) have not taken into account in their model that calcium interacts with phospholipid bilayers. Such interactions induce changes in lipid dynamics, structure and affinity of phospholipid-binding proteins (32).

AP complexes are targeted to their respective membrane via binding to specific phosphoinositides, AP-1 to PI-4-P and AP-2 to PI-4,5-P_2. There are no data in the literature describing inhibition of a sorting motif/AP complex binding by basic residues located in the vicinity of the sorting motif (23, 25, 26). In fact, PI-4-P incorporated into liposomes binds AP-1 and the interaction between adaptins and sorting motif-containing proteins is further stabilized by phospholipids during the polymerization of the vesicle coat (33). These results speak in favor of role of phospholipids and phosphoinositides in the interaction of AP-1 with its binding partners and vesicular assembly.

The μ2 adaptin of the plasma membrane AP-2 complex collects proteins for clathrin-mediated endocytosis (CME) and many proteins are transported by AP-1 as well as AP-2 CCV (17,18). μ2 was not isolated in our screen, despite the fact that it is highly homologous to μ1A. Therefore, we asked why μ2 could not bind L-selectin tail. This is likely explained by the fact that the key amino acids in μ1A involved in L-selectin tail binding are not found in μ2. Indeed, only the first glutamic acid residue of the L-selectin binding motif in μ1A is conserved in μ2 (E311>E321, E337>S347, E340>A350, D417>I430). Also the asparagine residues 318 and 338 and glutamine 419 of μ1A are not conserved in μ2, where they are replaced by leucine and glutamic acid residues. These data strongly indicate that the AP-2 complex, which is highly homologous to the AP-1 complex, does not play a role in the regulation of L-selectin cell surface expression.

We tested for L-selectin and AP-1 colocalization *in-vivo* by confocal microscopy. Besides the plasma membrane pool of L-selectin, we detected an intracellular pool of L-selectin localized next to AP-1 decorated domains of the TGN. This indicated that AP-1-dependent L-selectin sorting might be responsible for the formation of this putative L-selectin reserve pool.

Murine L-selectin tail contains one unique serine residue at position 364, which is conserved in human L-selectin. It was shown that upon stimulation of lymphoblastoid cell lines with PMA or chemoattractants, L-selectin tail becomes rapidly phosphorylated (16) and this is likely caused by recruitment of PKC family members to L-selectin tail (14). However, little is known on the relationship between L-selectin tail phosphorylation and its interaction with binding partners *in vivo*. We found low amounts of μ1A associated with anti L-selectin immunoprecipitates in resting Raw 264.7 macrophages. However, this association increased when the cells were stimulated with PMA. This result could be explained in at least two ways, involving phosphorylation of both μ1A and/or L-selectin tail. One phosphorylation step would be the enhanced phosphorylation of μ1A in response to PMA, because such phosphorylation would augment its interaction with L-selectin tail. Indeed, the cytoplasmic AP-1 complex is present in a closed conformation, in which the cargo binding domains of μ1A and σ1A are blocked by γ1 and β1 adaptins. The complex has to undergo a conformational change to an 'open' state. This causes the release of the μ1A C-terminal domain from the large adaptins, enabling its movement towards the membrane and the cytoplasmic sorting domains of membrane proteins. The open conformation is favoured by threonine phosphorylation of μ1A in the flexible linker region connecting the N- and C-terminal domains (34-36). Such a mechanism of activation has also been described for the L-selectin tail interacting protein moesin. Phosphorylation of moesin by PKCα on a threonine residue located in the C-

terminal part unfolds the protein, thus allowing it to interact with L-selectin tail (37).

The second phosphorylation reaction to explain a PKC-stimulated association between μ1A and L-selectin tail would be the L-selectin tail phosphorylation. However, μ1A does not bind the phospho-L-selectin tail peptide and this is due to the fact that the phosphate group on serine 364 is in close proximity to the negatively charged residues E311 and E340 of μ1A. In keeping with our results, other investigators showed inhibition of calmodulin binding to the L-selectin-p-tail *in vitro* and in intact THP-1 cells (6).

The AP-1 coat is found on the TGN and on early endosomes (EE) and it mediates constitutive protein transport from the EE to the TGN as well as from the TGN to EE as exemplified by the Mannose-6-phosphate receptors, Furin or Sortilin (20, 21, 26). Interestingly, beside its role as cargo binding and constitutive regulator of protein transport, the role of AP-1 in regulated secretory pathways has also been shown (38-40). Our data showing that phosphorylation of both μ1A and L-selectin tail control the interaction between these two proteins speaks in favour of a regulated, rather than a constitutive AP-1-dependent secreted pathway for L-selectin. We developed a model of AP-1-dependent L-selectin sorting based on our data and the known functions of AP-1 (Fig. 7B). We propose that stimulation of μ1A phosphorylation activates AP-1 and enhances AP-1-dependent protein transport from the TGN to EE. Phosphorylation of L-selectin tail in activated leukocytes would prevent its binding to μ1A (AP-1) in EE leading to less incorporation of L-selectin into AP-1 CCV for retrograde EE to TGN transport. Consequently, L-selectin would transiently accumulate in EE from which it can be readily transported to the plasma membrane through exocytosis (21). Such an indirect function of AP-1 in the regulation of plasma membrane protein transport has been demonstrated by us for both mannose-6-phosphate receptors in mouse fibroblasts (20, 21). Thus large amounts of L-selectin would be exported to the plasma membrane and could be readily clustered on microvilli relevant for leukocyte rolling and adhesion. We are currently investigating this working hypothesis of stimulation-dependent compartment specific L-selectin tail and μ1A phosphorylation in leukocytes.

In summary, we have made several novel and important observations regarding L-selectin functions. First, we identified μ1A as a novel protein interacting with L-selectin tail. Second, we demonstrated colocalization of an intracellular pool of L-selectin with AP-1. Third, a novel binding motif formed by a triplet of di-basic amino acid motifs in L-selectin (RRxKKxKK) mediates this interaction. Fourth, L-selectin tail phosphorylation inhibits μ1A binding. From these data we can conclude that AP-1, but not AP-2, is involved in the transport pathway regulating L-selectin secretion to the plasma membrane upon stimulation.

EXPERIMENTAL PROCEDURES

Antibodies—The antibodies and their sources were as follows: anti-L-selectin Ab lam 1-116 (sc-13505) was purchased from Santa-Cruz Biotechnology (Heidelberg, Germany), the mouse IgG2a isotype control Ab (UPC-10), was purchased from Sigma-Aldrich (Munich, Germany), the anti-μ1A Ab (ab 170277) was bought from abcam (Cambridge, UK), the anti-GST mouse monoclonal Ab (ref: 71097-3) was from Novagen/Merck (Darmstadt, Germany).

Chemicals—Protease inhibitor tablets were from Roche (Penzberg, Germany), the phosphatase inhibitor cocktail 1 and 2 were purchased from Sigma-Aldrich (Munich, Germany). Dynabeds Myone Streptavidine C1 and Mammalian Protein Extraction Reagent (MPER) buffer were from Invitrogen/Thermo Fischer Scientific (Karlsruhe, Germany), Protein A sepharose TM CL-4B and streptavidine sepharose beads were purchased from GE Healthcare Life Sciences (Munich, Germany). RPMI 1600 medium was purchased from GIBCO/Thermo Fischer Scientific (Karlsruhe, Germany). Fetal bovine serum and Octyl-beta-Glucoside were from Sigma-Aldrich (Munich, Germany). TAPI-O was from Peptides International (Louisville, USA).

Peptides—Automated solid phase synthesis of peptides using Fmoc/tBu-chemistry was carried out at the Biochemistry core facility of the Max Planck Institute for Biochemistry

(Martinsried, Germany). Characterization and quality control (purity) was done by RP-HPLC. All peptides were desthiobiotinylated and a spacer (hexanoic acid or Ahx) between the desthiobiotin group and the first amino acid of the peptides was introduced. The following peptides were used in this study:
Desthiobiotin-GAhxEGRMKQPDKRDSKLKRY-OH (scrambled peptide);
Desthiobiotin-GAhxRRLKKGKKSQERMDDPY-OH (L-selectin tail)
Desthiobiotin-GAhxRRLKKGKKS$_p$QERMDDPY-OH (L-selectin-p-tail)
Desthiobiotin-GAhxRRLKKGKKDQERMDDPY-OH (L-selectin S364D tail)
Desthiobiotin-GAhxRALKKGKKSQERMDDPY-OH (L-selectin R357A tail)
Desthiobiotin-GAhxRRLKAGKKSQERMDDPY-OH (L-selectin K360A tail)
Desthiobiotin-GAhxRRLKKGKASQERMDDPY-OH (L-selectin K363A tail)
Desthiobiotin-GAhxRRLKKGKKSQERMNDPY-OH (L-selectin D369N tail)
Desthiobiotin-GAhxRRLKKGKKSQERMDNPY-OH (L-selectin D370N tail)
Desthiobiotin-GAhxRRLKKGKKSQERM|NNPY-OH (L-selectin D369N, D370N tail)

Cell culture—Raw 264.7 macrophages were grown in 75 cm^3 flasks at 37°C in an atmosphere of 5 % CO_2 in RPMI 1600 medium supplemented with 10% FCS, antibiotics, and L-glutamine (20 mM).

Immunoprecipitation and Western blotting—Raw 264.7 macrophages were pretreated with the sheddase inhibitor TAPI-O (10 min, 10 μg/ml) and then incubated in the absence or presence of PMA (1 μg/ml) for different time periods. Thereafter, cells were collected by centrifugation (200 g, 2 min) and washed with PBS. The tubes were put on ice for 5 min after which 1 ml of lysis buffer was added. The lysis buffer consisted of: 50 mM Tris-HCl, pH 7.4, 30 mM Octyl-beta-Glucoside, 1 mM EDTA, 110 mM NaCl, protease inhibitors and phosphatase inhibitors. Cell lysates were clarified by centrifugation and the supernatants were subjected to immunoprecipitation. This was performed by exposure to the anti-L-selectin Ab (1 μg/ml) or the isotype-matched IgG2a Ab (1μg/ml) for 1 h, and then to 40 μl of a 50% slurry of protein A-Sepharose for 1h. The beads were subsequently collected by centrifugation (200 g, 1 min) and washed 3 times with lysis buffer. The beads were then re-suspended in 2x concentrated Laemmli sample buffer and boiled under reducing conditions (1 mM DTT) for 5 min. The immunoprecipitated proteins were subjected to electrophoresis on 10% SDS-PAGE and transferred to polyscreen PVDF transfer membranes. The membranes were blocked in TBS supplemented with 0.2 % Tween-20 and 3% fat-free skimmed milk and then incubated for 1h with a primary antibody (1:500 dilution of the rabbit anti-μ1A Ab (19); or 1 μg/ml dilution of the mouse anti-L-selectin Ab) and thereafter washed 3 times for 5 min in TBS supplemented with 0.2% Tween. The membranes were subsequently incubated for 1h with peroxidase-conjugated goat anti-rabbit IgGs (1:10 000) or goat anti-mouse IgGs (1:10 000) in TBS supplemented with 0.2% Tween-20. The blots were washed 3 times for 5 min with TBS supplemented with 0.2% Tween-20 and antibody binding was visualised by enhanced chemiluminesense (ECL).

Peptide pulldown assays—L-selectin peptides were dissolved in PBS/0.01% Tween-20 and then 20 μl of Streptavidine sepharose beads were added for 1h followed by the addition of 2% BSA for 30 min. The beads were collected by centrifugation (200 g, 1 min), and washed once with PBS/0.01% Tween-20.
L-selectin tail peptides coupled to Streptavidine sepharose beads were then incubated in 1ml of cold buffer composed of 50 mM Tris-HCl, pH 7.4, 0.5% CHAPS, 110 mM NaCl. 50 ng purified μ1A expressed as GST fusion proteins was added. The Eppendorf tubes were rotated for 1h in the cold room after which pellets were collected by centrifugation (200 g, 1 min). The pellets were washed 3 times with washing buffer (50 mM Tris-HCl, pH 7.4, 0.5% CHAPS, 300 mM NaCl). The beads were subsequently re-suspsended in 2x concentrated Laemmli sample buffer and boiled under reducing conditions for 5 min. The proteins were subjected to electrophoresis on 10% SDS-PAGE and transferred to polyscreen PVDF

transfer membranes and Western blot analysis was carried out as described above using a rabbit anti-μ1A Ab or an anti-GST Ab.

L-selectin peptides coupled to streptavidine sepharose beads were also incubated with lysate extracts of Raw 264.7 macrophages. Briefly, the cells were lysed in buffer composed of 50 mM Tris-HCl, pH 7.4, 0.5% CHAPS, 1 mM EDTA, 110 mM NaCl, protease and phosphatase inhibitors. Lysates were then added for 1h to L-selectin tail peptides coupled to beads. Thereafter, the beads were collected by low speed centrifugation (200 g, 1 min), washed 3 times with lysis buffer and finally re-suspended in 2x concentrated Laemmli sample buffer and boiled under reducing conditions. μ1A bound to L-selectin tail was detected by Western blot analysis as described above.

Proteomics analysis—Raw 264.7 macrophages were stimulated for 1 min with PMA (1 μg/ml) after which the cells were pelleted, and washed with ice cold PBS. The cells were subsequently lysed with MPER buffer. Lysates were then incubated with Dynabeds Myone Streptavidine C1 which have been coupled to either L-selectin tail peptide or scrambled peptide (see above). After 2h incubation in the cold room under rotation, the beads were collected with a magnet, and washed gently three times with MPER buffer. The beads were then re-suspended in 50 mM Tris-HCl, pH 8.0 containing 10 mM biotin to dissociate the biotin analogue desthiobiotin from streptavidin beads. After 1h in the cold room, supernatants were collected, and proteins were precipitated overnight at -20^{o} C with 100 % acetone. Proteins were pelleted by centrifugation (15,000g, 15 min) in a cold centrifuge. Subsequently, the pellet was air dried under a fume hood. 20 μl of 6M Guanidium HCl containing 10 mM of the reducing agent tris(2-carboxyethyl)phosphine (TCEP) and 40 mM of the alkylation agent 2-chloroacetamine (CAA) were added to each pellet and the samples were boiled for 10 min. 180 μl of LT-Digestion buffer (25 mM Tris-HCL, pH 8.5 containing 10% acetonitrile) was added followed by 1 μg of each trypsin and Lys-C endopeptidase. The tubes were left overnight at 37^{0}C under shacking. Thereafter, 8μl of 25% trifluoroacetic (TFA) (1 % final) was added to stop the reaction. The peptides were bound to triple filter surface (Ref: SDB-RPS 3X), and after washing the filters 3 times with 0.2% TFA, proteins were eluted with 60 μl buffer containing 80% acetonitrile and 5% ammonium. Peptides were analysed on a LTQ-Orbitrap analyser, identified and quantified using the MAxQUant software (41).

Purification of GST Fusion Proteins—Expression of μ1A as GST fusion proteins has been described in detail (42). Briefly, pGEX-5X3 plasmids were transformed into *E. coli* BL21. 1 mM Isopropyl β-d-thiogalactopyranoside was added overnight to bacterial cultures grown at 26 °C. Bacteria were harvested, put on ice, washed once with ice cold PBS and lysed by the addition of a buffer made-up 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 110 mM NaCl, and protease inhibitors. This was followed by sonication. The lysates were clarified by centrifugation (10,000 g, 15 min), supernatants were collected, and 10 % glycerol was added. These supernatants were incubated in the cold room with glutathione-Sepharose beads pre-equilibrated in PBS buffer. After 1h, the beads were collected by centrifugation (200 g, 1 min), and washed 3 times with ice cold PBS. The GST fusion proteins were eluted by adding 200 μl of buffer containing 50 mM Tris-HCl, pH 8.0 and 10 mM reduced glutathione. Eluted solutions were passed through a G-25 sephadex column which was subjected to centrifugation (500 g, 1 min) and GST-fusion proteins free of glutathione were collected. Protein concentrations were estimated by spectrometry.

Immunofluoresence confocal microscopy— THP-1 cells stably expressing wild type L-selectin tagged to green fluorescent protein (WT L-selectin-GFP) were generated and cultured as described previously (6). Cultured THP-1 cells were harvested and resuspended in warmed neat RPMI at a density of 0.5 x 10^{6}cells per ml. Cells were subsequently seeded onto poly-L-lysine-coated coverslips and allowed to settle for 10 min at 37^{o}C. RPMI was aspirated and coverslip-bound cells were fixed in 3% paraformaldehyde for 20 min at room temperature. Cells were washed three times in PBS and then permeabilised in 0.6% saponin for 10 min at room temperature. After washing fixed cells with PBS, cells were blocked in 5% foetal calf serum containing Fc receptor

block (Miltenyi Biotec Ltd) for 1 hour at room temperature before staining with 1:300 (v/v) of 0.1 mg/ml TRITC-phalloidin and anti-γ1 AP-1 mouse monoclonal antibody (250 μg/mL, Becton Dickinson) diluted at 1:75 (v/v) overnight at 4°C. Cells were washed free from excess phalloidin and antibody, blocked as before and then stained with secondary goat anti-mouse (Life Technologies) Alexa Fluor 633 (1:400 dilution). Specimens were mounted onto glass slides using mounting medium (Dako). Images were acquired using Leica SP5 confocal microscope, using x63 objective lens. Scale bar = 7.5 μm.

Molecular Modelling—Molecular docking of L-selectin tail to μ1A was conducted using the HEX 8.0 protein-protein docking program (43). The crystal structure of μ1A (PDB code: 4P6Z) and the structural model of 17-residue long L-selectin tail modelled using the crystal structures of the eight residue long L-selectin tail (PDB code: 2LGF) were used. The remaining eight amino acid residues of the L-selectin tail were modelled using the loop prediction option of the Prime module within Schrödinger software (44). The L-selectin tail in a wild type as well as mutated forms (S364pS, S364D, D369N and D370N) were used for docking to facilitate a choice of docking solutions. The μ1A/L-selectin binding surface interactions were mapped based on shape and electrostatics complementarities as implemented in HEX 8.0. Default settings for other docking parameters have been used. HEX 8.0 has generated over 100 solutions for each docking run. Besides the Hex energy function, the obtained docking pose of the L-selectin tail in the wild type and mutated forms was evaluated based on its ability to explain the mutagenesis data. In addition, only docking poses on the surface of μ1A, which is not in an interaction with other subunits within AP-1 were considered.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHORSHIP CONTRIBUTIONS

KD designed the project, performed the research, analyzed the data and wrote the manuscript; IT performed the modelling of μ1a-L-selectin tail interaction, analyzed the data, and wrote part of the manuscript; AI performed the colocalization studies; PS provided scientific expertise, analyzed the data, and wrote part of the manuscript. All authors read and approved the final manuscript.

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FOOTNOTES

Footnote to the title

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[1] The abbreviations used are: Abs, antibodies; CAA, 2-chloroacetamine; CCV, clathrin-coated vesicles; DTT, 1,4 dithiothreitol; EE, early endosomes; GST, glutathione S-transferase; Mammalian Protein Extraction Reagent, MPER; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol myristate acetate; TBS, Tris-buffered saline; TCEP, tris(2-carboxyethyl)phosphine); TFA, trifluoroacetic. 2 adaptin; TGN, trans-Golgi-network.

FIGURE LEGENDS

Figure 1: Identification of μ1A as a novel protein interacting with L-selectin cytoplasmic tail
A, peptide sequences used to identify proteins interacting with L-selectin cytoplasmic tail.
B, Raw 264.7 macrophages were stimulated for 1 min with PMA (1 μg/ml) after which cells were lysed. Lysates were incubated with synthetic desbiotinylated peptides (scrambled control or L-selectin tail peptides) immobilized on Dynabeads Myone Streptavidin. Proteins bound to the peptides were eluted, acetone precipitated and digested with trypsin and Lys-C. Quantitative mass spectrometric analysis was performed on a LTQ Orbitrap mass spectrometer and analysed using the MaxQuant Software. Peptides deriving from the bait (Sell for L-selectin) and L-selectin binding proteins are indicated on the graph. The AP1m1 gene encodes for the μ1A subunit of the AP-1 complex.

Figure 2: μ1A binds L-selectin cytoplasmic tail peptide *in vitro*
(A), BL21 bacteria were transformed with pGEX-5X3 plasmids encoding for the different GST forms of μ1A proteins including full length μ1A (GST-FL- μ1A), the N-terminal (GST-N-μ1A) or the C-terminal part of μ1A (GST-C-μ1A). After induction with IPTG, bacteria were collected, lysed, and lysates incubated with glutathione sepharose beads. After 1h, the beads were collected, washed, and 2x Laemmli buffer containing 1 mM DTT was added. Samples were run on a 10% SDS-PAGE and proteins were stained with coomassie blue. The position of GST-FL-μ1A, GST-N-μ1A, GST-C-μ1A, or GST is indicated on the left hand side by arrows.
(B), Purified GST-FLμ1A or GST were incubated with a scrambled peptide (sc peptide) or L-selectin cytoplasmic tail peptide (L-sel tail peptide) coupled to streptavidin-coated beads. After 1h, the collected beads were washed, and re-suspended in Laemmli buffer. The proteins were separated on a 10% SDS-PAGE, transferred onto a PVDF membrane which was subjected to Western blot analysis using an anti-GST Ab (top panel). The position of GST-FL-μ1A or GST is indicated on the left hand side by arrows. Loading controls (which correspond to the amount of GST-FL-μ1A or GST used for the peptide pull down assay) are shown in the panel below. Note that an excess of GST versus GST-FL-μ1A has been used.
(C) Binding of GST-FL-μ1A, GST-C-μ1A or GST to a scrambled peptide (sc peptide) or L-selectin tail peptide (L-sel tail peptide) was carried out as described in section B above.
(D), Binding of GST-FL-μ1A, GST-C-μ1A or GST to a scrambled peptide (sc peptide) or L-selectin tail peptide (L-sel tail peptide) was carried out as described in section B & C above with the exception that Western blot analysis was carried out with an anti-μ1A Ab.

Figure 3: μ1A is associated with anti-L-selectin immunoprecipitates
Raw 264.7 macrophages, stimulated or not with PMA (1 μg/ml) for different time periods were lysed and lysates subjected to immunoprecipitation with an anti-L-selectin Ab (A) or a control IgG2a (B). After 1h, protein A-sepharose beads were added for an additional hour. The beads were then collected by centrifugation, washed, and re-suspended in 2 x Laemmli buffer. The immunoprecipitates were subjected to 10% SDS-PAGE, transferred to a PVDF membrane, which was cut into two pieces. The lower part of the membrane was immunoblotted with an anti-μ1A Ab (A & B, top panels) and the top part of the membrane was immunoblotted with an anti-L-selectin Ab (A & B, lower panels), as described in Materials and Methods. The arrows indicate the position of μ1A, L-selectin, or IgG.

Figure 4: Subcellular distribution of L-selectin and AP-1 in monocyte-like THP-1 cell line
THP-1 cells stably expressing wild type L-selectin, C-terminally tagged with green fluorescent protein (WT L-selectin-GFP), were prepared for confocal microscopy as described in the experimental procedures. Cells were labeled for endogenous AP-1 (anti-γ1 subunit, blue) and actin (TRITC-phalloidin: red). a) L-selectin (green) is present at the plasma membrane and

within discrete punctae that colocalize with AP-1-positive signal within the TGN. b) the rectangles show the areas used to perform quantification of L-selectin/μ1A colocalization. A region of interest of similar dimension was used to measure intracellular regions and at the plasma membrane. c) the volocity software was used to measure the overlap coefficient between blue and green signals corresponding to AP-1 and L-selectin, respectively. Each signal was thresholded to remove background signals prior to analysis. Threshold parameters were maintained in all of the analysis. Instrument parameter settings remained constant between image acquisitions. The data represents means of colocalization co-efficiency ± SEM of 22 cells prepared over three independent experiments. $p < 0.0001$ (Unpaired Student's t-test). Scale bar = 7.5 μm.

Figure 5: Phosphorylation of serine 364 of L-selectin cytoplasmic tail prevents the binding of μ1A
(A), purified GST-μ1A or GST-C-μ1A were incubated with a scrambled peptide (sc peptide), L-selectin tail (L-selectin tail peptide), a phosphorylated L-selectin tail peptide (L-sel-p-peptide) or the L-selectin tail peptide in which serine 364 was replaced by an aspartic acid (L-sel S364D peptide). Thereafter, association of GST-μ1A (A, left panel) or GST-C-μ1A (A, right panel) with the different L-selectin tail peptides were determined as described in Figure 2.
(B), purified GST-μ1A or GST-C-μ1A were incubated with a scrambled peptide (sc peptide), L-selectin tail (L-selectin tail peptide), or L-selectin tail peptide in which serine 364 was replaced by an alanine residue (L-sel S364A peptide). Thereafter, association of GST-μ1A (B, left panel) or GST-C-μ1A (B, right panel) with the different peptides was measured as described in the section A above.

Figure 6: Replacement of arginine or lysine residues within the clusters of di-basic residues prevents binding of μ1A
(A), Amino acid sequence of L-selectin cytoplasmic tail and the different substitutions in the 3 clusters of di-basic residues is shown.
(B), purified GST-μ1A or GST-C-μ1A were incubated with a scrambled peptide (sc peptide), L-selectin cytoplasmic tail (L-selectin tail peptide), or peptides in which one amino acid within the clusters of di-basic residues were replaced by alanine residues. These include L-sel R357A peptide, L-sel R360A peptide or L-sel R363A peptide. Thereafter, association of GST-μ1A (B, left panel) or GST-C-μ1A (B, right panel) with the different peptides was determined as described in Fig. 2D.
(C), Amino acid sequence of L-selectin cytoplasmic tail with substitutions of aspartic acid residues (D) by asparagine (N) is indicated.
(D), purified GST-C-μ1A was incubated with a scrambled peptide (sc peptide), L-selectin tail (L-sel tail peptide), or peptides in which one or two of the aspartic acid residues (D) was replaced by an asparagine (N) residue. These include L-sel D3369N peptide, L-sel D370N peptide or the double substituted L-sel D369N, D370N peptide. Thereafter, association of GST-C-μ1A with the different peptides was determined as described above.

Figure 7: Molecular model of L-selectin-μ1A interactions
(A, left panel), L-selectin tail docked to the surface of μ1A. L-selectin tail is in orange. The surface of μ1A is coloured based on the electrostatic potential, the red and blue colours show negatively and positively charged areas, correspondently. Residues of L-selectin tail used in mutagenesis are labeled.
(A, right panel), Binding interactions between L-selectin tail and μ1A. Hydrogen bonds are in a black dotted line. Residues of L-selectin tail and μ1A are labeled in red and black, respectively.
(B), Model for the AP-1-dependent L-selectin sorting in leukocytes at rest (left) and upon stimulation (right). Pi indicates the addition of inorganic phosphate to the L-selectin tail and μ1A. Bold letters indicate a higher phosphorylation rate of μ1A in stimulated leukocytes compared to cells at rest. Bold arrows indicate enhanced TGN-to-endosome L-selectin transport by AP-1 due to phosphorylation of μ1A

and thus more activated AP-1 complexes. Phosphorylation of L-selectin tail on serine 364 in EE causes the transient accumulation of L-selectin in EE, because this L-selectin cannot bind μ1A, leading to its exocytosis to the plasma membrane (PM).

Figure 1

A

L-selectin tail peptide: Desthiobiotin-GAhxRRLKKGKKSQERMDDPY-OH

Scrambled control peptide: Desthiobiotin-GAhxEGRMKQPDKRDSKLKRY-OH

B

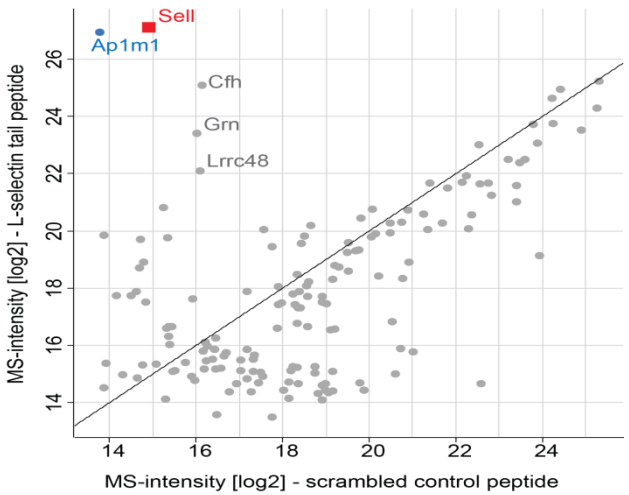


Figure 2

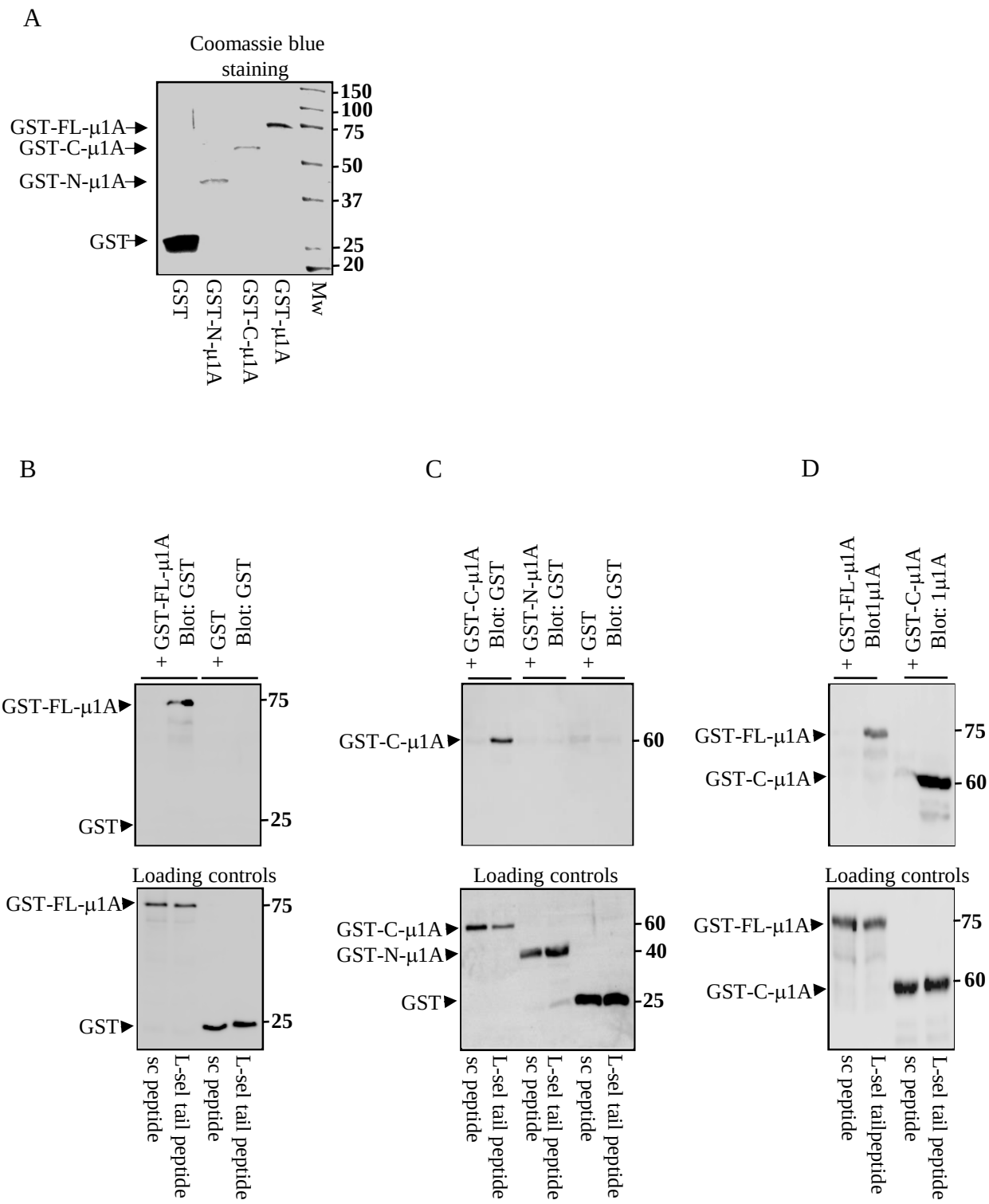

A
Coomassie blue staining
GST-FL-µ1A
GST-C-µ1A
GST-N-µ1A
GST
150
100
75
50
37
25
20
GST
GST-N-µ1A
GST-C-µ1A
GST-µ1A
Mw
B
+ GST-FL-µ1A Blot: GST
+ GST Blot: GST
GST-FL-µ1A
GST
75
25
Loading controls
GST-FL-µ1A
GST
75
25
sc peptide
L-sel tail peptide
sc peptide
L-sel tail peptide
C
+ GST-C-µ1A Blot: GST
+ GST-N-µ1A Blot: GST
+ GST Blot: GST
GST-C-µ1A
60
Loading controls
GST-C-µ1A
GST-N-µ1A
GST
60
40
25
sc peptide
L-sel tail peptide
sc peptide
L-sel tail peptide
D
+ GST-FL-µ1A Blot1µ1A
+ GST-C-µ1A Blot: 1µ1A
GST-FL-µ1A
GST-C-µ1A
75
60
Loading controls
GST-FL-µ1A
GST-C-µ1A
75
60
sc peptide
L-sel tailpeptide
sc peptide
L-sel tail peptide

Figure 3

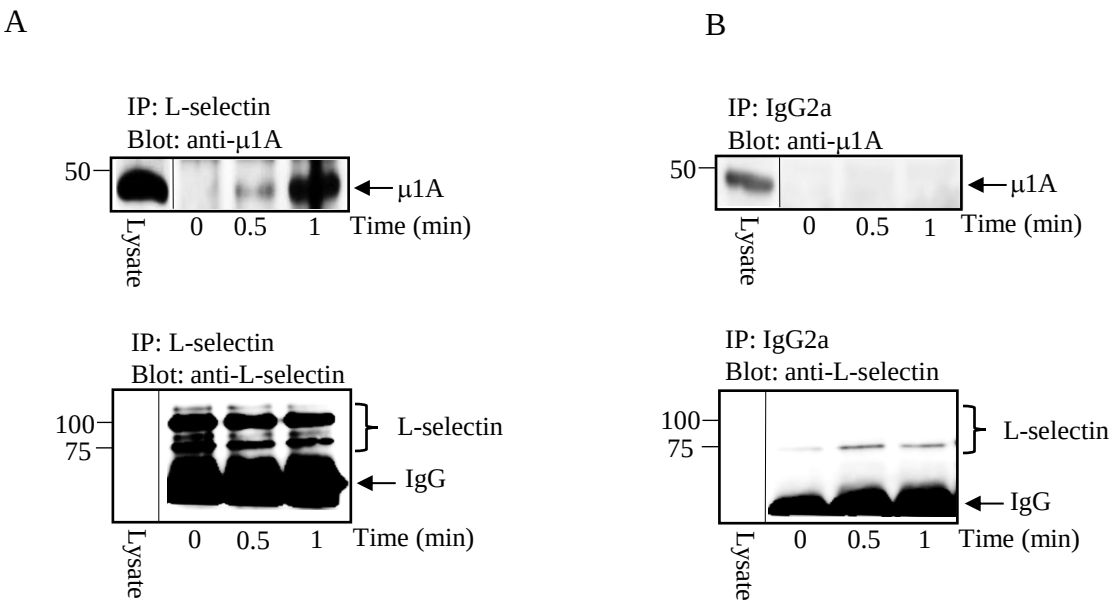
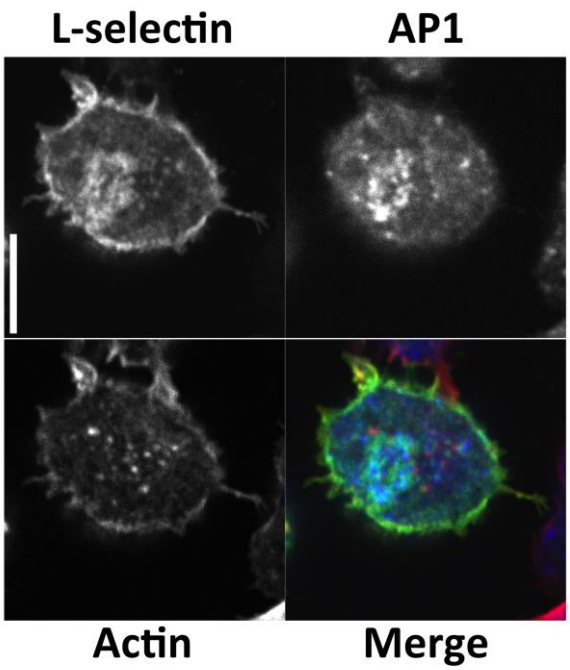
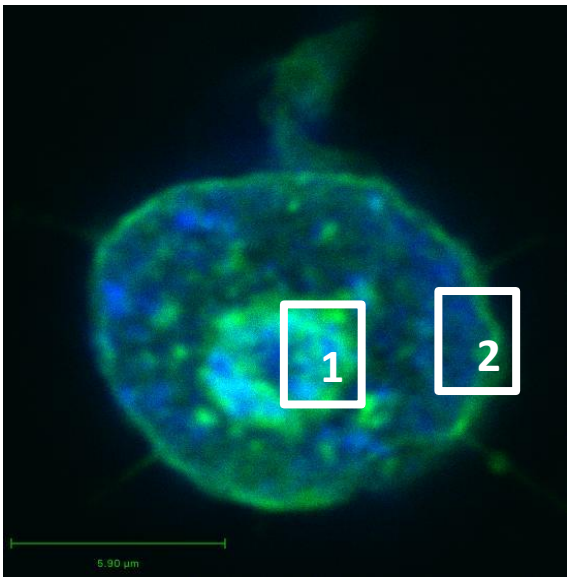

A
IP: L-selectin
Blot: anti-μ1A
50
μ1A
Lysate
0
0.5
1
Time (min)
IP: L-selectin
Blot: anti-L-selectin
100
75
L-selectin
IgG
Lysate
0
0.5
1
Time (min)
B
IP: IgG2a
Blot: anti-μ1A
50
μ1A
Lysate
0
0.5
1
Time (min)
IP: IgG2a
Blot: anti-L-selectin
100
75
L-selectin
IgG
Lysate
0
0.5
1
Time (min)

Figure 4

a


L-selectin
AP1
Actin
Merge

b


1
2
5.90 µm

c

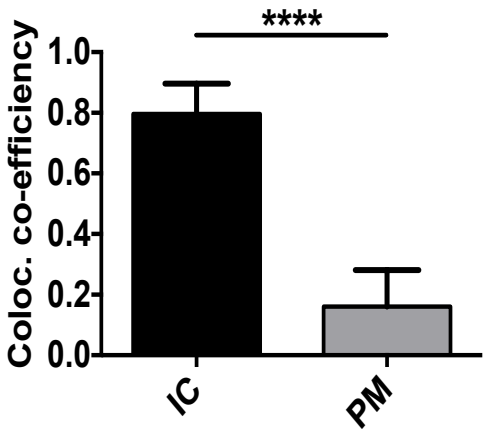

****
Coloc. co-efficiency
1.0
0.8
0.6
0.4
0.2
0.0
IC
PM

Figure 5

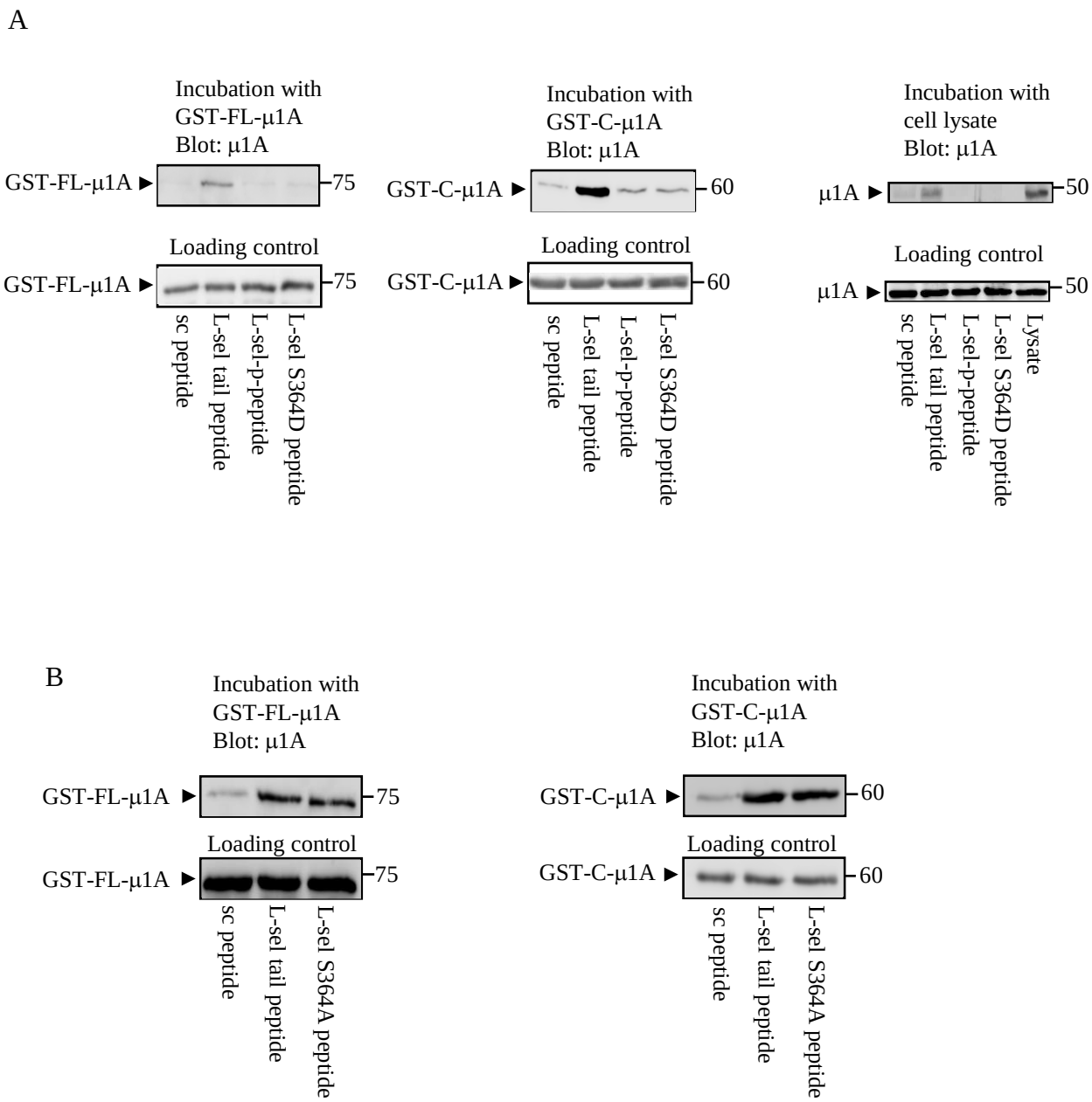

A
Incubation with
GST-FL-µ1A
Blot: µ1A
GST-FL-µ1A ▶
75
Loading control
GST-FL-µ1A ▶
75
sc peptide
L-sel tail peptide
L-sel-p-peptide
L-sel S364D peptide
Incubation with
GST-C-µ1A
Blot: µ1A
GST-C-µ1A ▶
60
Loading control
GST-C-µ1A ▶
60
sc peptide
L-sel tail peptide
L-sel-p-peptide
L-sel S364D peptide
Incubation with
cell lysate
Blot: µ1A
µ1A ▶
50
Loading control
µ1A ▶
50
sc peptide
L-sel tail peptide
L-sel-p-peptide
L-sel S364D peptide
Lysate
B
Incubation with
GST-FL-µ1A
Blot: µ1A
GST-FL-µ1A ▶
75
Loading control
GST-FL-µ1A ▶
75
sc peptide
L-sel tail peptide
L-sel S364A peptide
Incubation with
GST-C-µ1A
Blot: µ1A
GST-C-µ1A ▶
60
Loading control
GST-C-µ1A ▶
60
sc peptide
L-sel tail peptide
L-sel S364A peptide

Figure 6

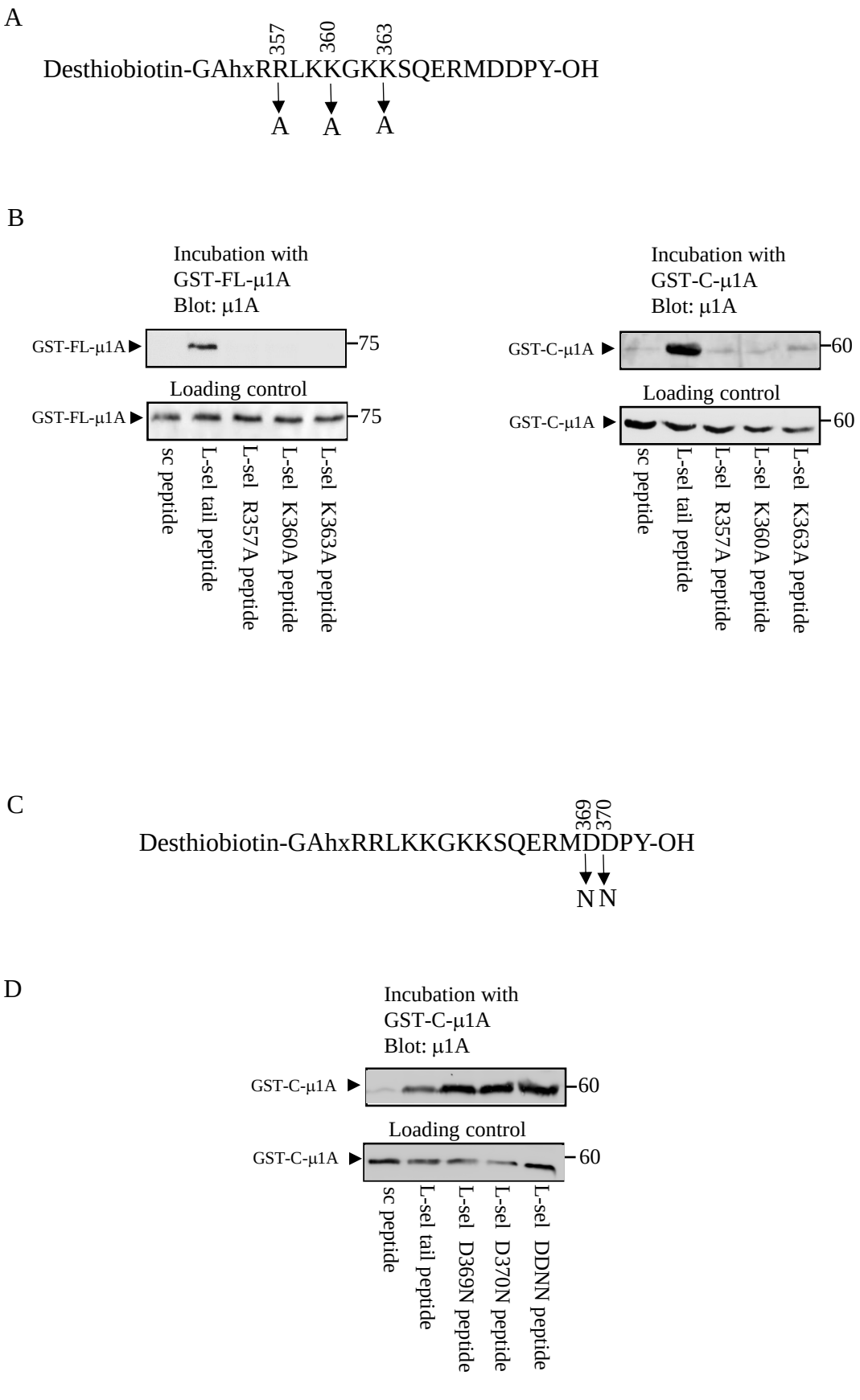
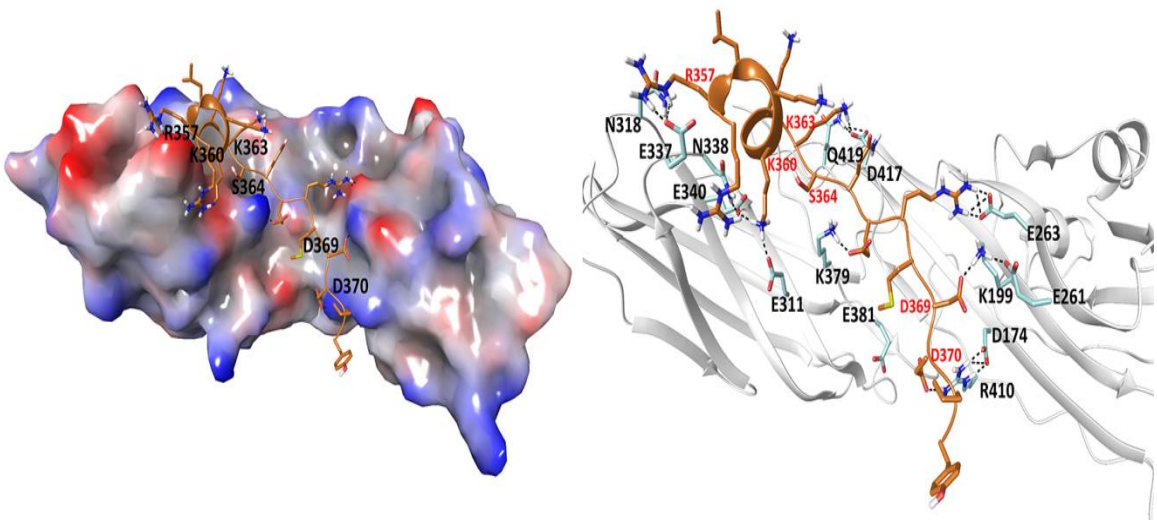
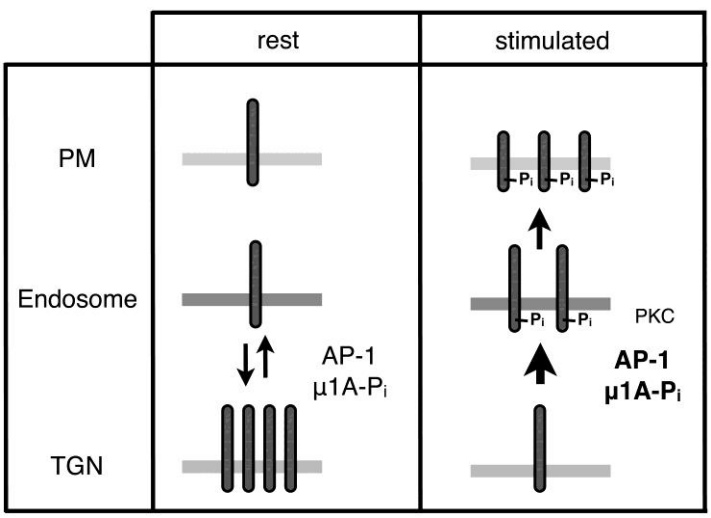

A
357
360
363
Desthiobiotin-GAhxRRLKKGKKSQERMDDPY-OH
A A A
B
Incubation with
GST-FL-µ1A
Blot: µ1A
GST-FL-µ1A
75
Loading control
GST-FL-µ1A
75
sc peptide
L-sel tail peptide
L-sel R357A peptide
L-sel K360A peptide
L-sel K363A peptide
Incubation with
GST-C-µ1A
Blot: µ1A
GST-C-µ1A
60
Loading control
GST-C-µ1A
60
sc peptide
L-sel tail peptide
L-sel R357A peptide
L-sel K360A peptide
L-sel K363A peptide
C
369
370
Desthiobiotin-GAhxRRLKKGKKSQERMDDPY-OH
N N
D
Incubation with
GST-C-µ1A
Blot: µ1A
GST-C-µ1A
60
Loading control
GST-C-µ1A
60
sc peptide
L-sel tail peptide
L-sel D369N peptide
L-sel D370N peptide
L-sel DDNN peptide

Figure 7

A


μ1A/L-selectin tail complex
R357
K360
K363
S364
D369
D370
N318
E337
N338
E340
K363
K369
Q419
D417
S364
E263
K379
E311
E381
D369
K199
E261
D174
D370
R410

B


rest
stimulated
PM
Endosome
TGN
AP-1
μ1A-Pi
Pi
PKC
AP-1
μ1A-Pi

The cytoplasmic tail of L-selectin interacts with the adaptor-protein complex AP-1 subunit μ1A via a novel basic binding motif

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